ELECTROPHORETIC ANALYSES OF SERUM PROTEINS OF THE ALBINO RAT[1, 2, 3]

DAVID F. McCORMICK AND JAMES D. GRAHAM

Department of Biology, Bowling Green State University, Bowling Green, Ohio 43403

ABSTRACT

Study of the leukemias and polycythemia has been complicated by the inability to isolate pure preparations of suspected "control factors" of hemopoiesis. The presence in rat serum of a factor controlling granulocyte maturation in rat bone-marrow has been demonstrated, but precise analysis has not been possible because of the lack of clear identification of rat-serum proteins. This study analyzes serum proteins of the albino rat through relative electrophoretic mobilities and specific staining.

Pooled rat- and human-serum samples were fractionated by gel filtration on Sephadex G–200. The resulting fractions were further separated by vertical discontinuous polyacrylamide gel electrophoresis. Identification of proteins was based on relative mobility and on reactions with general portein, glycoprotein, lipoprotein, and haptoglobin stains.

The low-molecular-weight fraction (MW 5,000–150,000) of rat serum contained prealbumin, albumin, three alpha-1, five alpha-2, and two beta globulins, and two alpha lipoproteins. The three alpha-1 globulins were implicated as a possible maturation factor. The slowest alpha-2 globulin was shown to be a haptoglobin. The intermediate-molecular-weight fraction (MW 150,000–300,000) was composed of two gamma globulins, a benzidine-positive alpha-2 globulin, two alpha-1 globulins, and an alpha lipoprotein component. The high-molecular-weight fraction (MW 300,000+) contained a beta-lipoprotein, two gamma macroglobulins, and two alpha-1 macroglobulins. Further diffuse staining was present in the gamma region. All of the non-lipoproteins in these two fractions were glycoproteins.

Mobilities of the major identifiable human-serum proteins (prealbumin, albumin, gamma globulin, haptoglobin) were similar to the same proteins in the rat, although many differences were apparent. Human albumin and prealbumin formed a separate fourth fraction which was not found in the rat.

INTRODUCTION

Study of the leukemias and polycythemia has been complicated by the inability to isolate pure preparations of suspected "control factors" of hemopoiesis. The

[1]Part of a thesis submitted to the Graduate School of Bowling Green State University in partial fulfillment of the requirements for the degree of Master of Arts.

[2]Supported in part by a National Science Foundation Institutional Grant to Bowling Green State University, by the Ohio Division, American Cancer Society, and by the Institute of Medical Research of the Toledo Hospital.

[3]Manuscript submitted July 30, 1970.

major obstacle to the study of granulopoietic control factors and their relation to leukemia is the absence of a positive identification of serum proteins in the albino rat. A factor controlling maturation of granulocytes in rat bone-marrow has been demonstrated in this laboratory and isolation and purification are being attempted (Graham, 1970; Graham and McMahon, 1971; Graham and Morrison, 1970).

Most identifications of serum proteins are based on those in human blood (Phelps and Putnam, 1960; Putnam, 1965; Sandor, 1966). There is a comparative lack of information on rat-serum proteins. Sandor (1966) reviewed early studies of rat serum using Tiselius (free) and paper electrophoresis with results yielding only the five basic components. He reported studies using immunoelectrophoresis, where rat serum showed a splitting of the gamma globulin line as a constant feature. As many as nineteen precipitin lines have been demonstrated in rat serum, including a prealbumin, an albumin, four alpha-1 globulins, six alpha-2 globulins, four beta globulins, and two gamma globulins. Two lipoproteins were among these, as was another band in the alpha-2 region that bound with hemoglobin and might thus correspond to the haptoglobin of human serum (Sandor, 1966). Starch-gel and agar electrophoresis gave eight protein components, as noted by Engle and Woods (1960).

Recent work on rat serum has been done using gel filtration and electrophoresis (Dolezalova, Brada, and Kocent, 1965). Sephadex gel filtration of normal serum followed by starch-gel electrophoresis of the resulting three fractions resolved as many as 17 zones. Immunoelectrophoresis of the fractions gave a total of 28 precipitin lines. Graham, Morrison, and Toepfer (1969), however, report only a total of 18 bands resulting from immuneolectrophoresis of similar gel filtration fractions. Dolezalova *et al.* (1965) also noted results for the specific detection of ceruloplasmin, haptoglobin, transferrin, and the lipoproteins.

These disagreements in the literature make necessary a thorough comparison of the proteins in rat serum with those found in human serum. The three serum-protein fractions resulting from gel filtration on Sephadex G–200 have been studied using vertical discontinuous polyacrylamide gel electrophoresis. After electrophoresis, each fraction was stained with a general protein stain, and the rat-serum fractions were also stained for glycoproteins, lipoproteins, and haptoglobin. Further, an attempt was made to indicate which bands might function as a "granulocyte maturation factor" (Graham and McMahon, 1971).

MATERIALS AND METHODS

Male Wisconsin Holtzman rats weighing between 190 and 200 grams and given food and water *ad libitum* were used as the source of rat serum which was taken from the abdominal aorta (Graham, 1969). Control human serum was collected by venipuncture from 10 adult males and was pooled.

Serum was fractionated using a 2.5×45-cm column packed with Sephadex G–200. The protein components were located by spectrophotometry at 280 mμ. The tubes containing each of the resulting protein fractions were pooled and concentrated with Sephadex G–25 (coarse), giving an approximate three-fold increase in optical density. The concentrated fractions were frozen and stored at −20°C.

Separation of the fractions of serum resulting from the gel filtration was further attained by a discontinuous method of vertical electrophoresis in an acrylamide gel. Prior to electrophoresis, the protein concentration of each fraction was determined using the Biuret test as described by Clark (1964). The fractions were then adjusted with the sample mixture to give a concentration of approximately 1 mg of protein per 100 μ of sample which was applied to each slot in the acrylamide gel.

The electrophoretic method was a modification of the recommended procedure for the separation of serum proteins (E–C Apparatus, 1966). Gel solutions were

made using a 95:5 ratio of acrylamide-bisacrylamide by weight. The 4-percent spacer gel was made up in a tris-HCl buffer, pH 6.7, 0.1 M; the 7-percent plug and running gel in a tris-HCl buffer, pH 8.9, 0.5 M. A tris-glycine buffer of pH 8.3, 0.04 M, was used in the electrode chambers. Electrophoresis was conducted at 200 volts and 10°C for 30 minutes to allow the sample to become completely stacked in the spacer gel. This was followed by an increase to 400 volts, a voltage which was maintained until the bromphenol blue front marker (Kohlrausch boundary) had reached a point 17.5 cm from the origin of application in the sample slots.

The acrylamide-gel slabs were treated with several staining procedures in order to identify specific components of the protein fractions. The general protain stain, amido black 10B (0.25%) in methanol-acetic acid-water (45:10:45), was used to label all proteins in each fraction. Glycoproteins were stained using the periodic acid-Schiff procedure described by Keyser (1964). Identification of the lipoproteins was done using the lipid crimson pre-staining method (Raymond, Miles, and Lee, 1966). Haptoglobin was identified using the benzidine stain (Ferris, Easterling, and Budd, 1963; Peacock, Bunting, and Queen, 1965).

RESULTS

The separation of rat serum by gel filtration on Sephadex G–200 is illustrated in Figure 1. Diagrammatic representations for each fraction of rat serum are shown in Figures 2, 3, and 4. A comparison of the fractions of rat and human serum is shown in Figure 5.

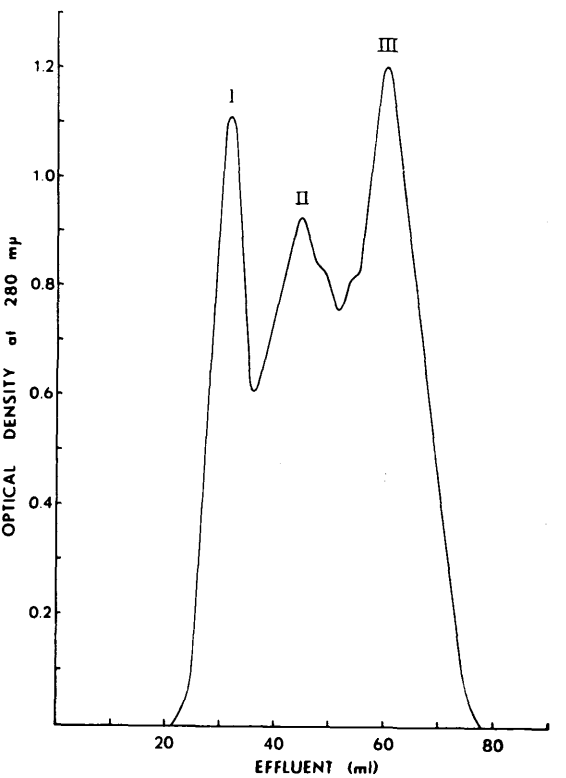

FIGURE 1. Sephadex G–200 separation of rat serum.

Areas between the major peaks contained proteins found in both neighboring peaks, but showed no unique bands upon electrophoresis. The contents of the two collector tubes closest to the top of the curve were considered to be representative of that fraction of serum and were pooled.

The rat high-molecular-weight fraction (peak I) showed six reproducible bands with general staining (AB in fig. 2). These included two alpha globulins, three bands in the gamma globulin region, and a band at the origin. The glycoprotein stain (GLY) showed positive staining in all these regions, and the lipoprotein stain (LIP) gave positive results at the origin. The glycoprotein nature of the alpha and gamma globulins was thus indicated, while the protein remaining at the origin was identified as a lipoprotein.

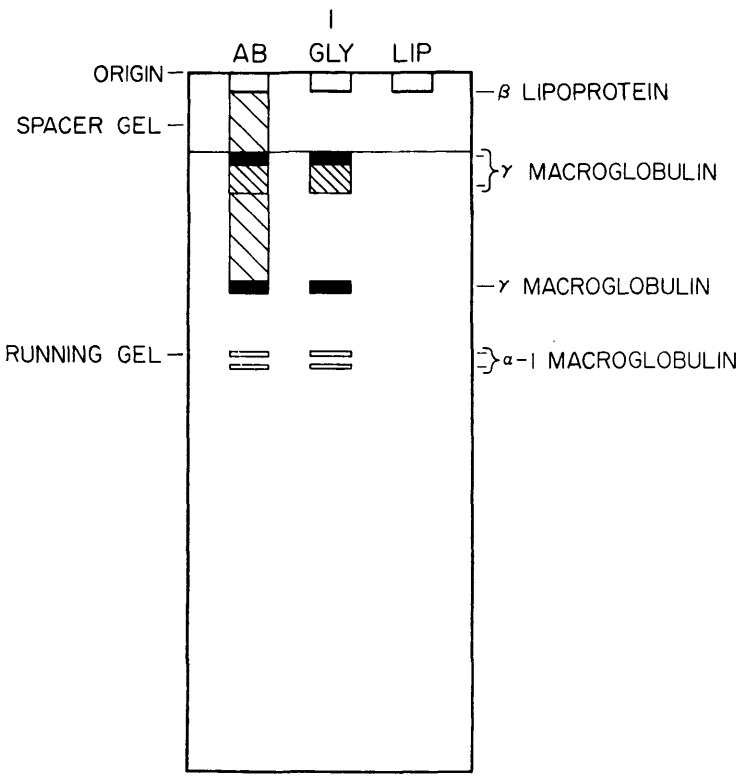

FIGURE 2. Diagrammatic representation of the high-molecular-weight fraction (I) of normal rat serum obtained by gel filtration when separated by vertical discontinuous polyacrylamide-gel electrophoresis.

The components of the rat intermediate-molecular-weight fraction (peak II) are seen in Figure 3. Two alpha globulins are followed by two gamma globulins in a diffusely stained region (AB). A band is also seen at the origin. Positive glycoprotein staining (GLY) is evident in all these bands, and the origin band also gives a positive lipoprotein reaction (LIP). A positive benzidine stain for haptoglobin is also shown in peak II (HP).

The low-molecular-weight fraction of rat serum (peak III) shows a number of reproducible bands (fig. 4). General staining (AB) indicates a prealbumin, an albumin, three alpha-1 globulins, five alpha-2 globulins, two beta globulins, and one band each at the origin and at the interface between the spacer and running gels. A positive glycoprotein stain (GLY) is found in the prealbumin region and

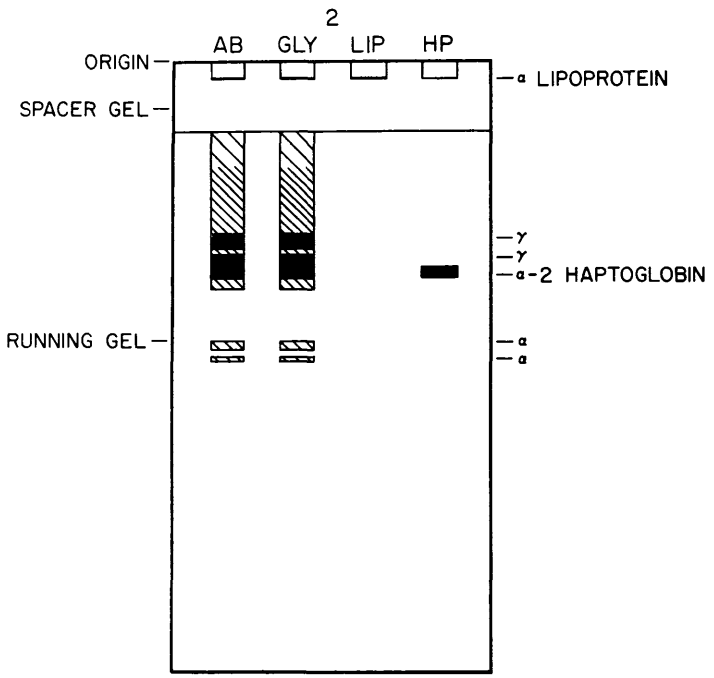

FIGURE 3. Diagrammatic representation of the intermediate-molecular-weight fraction (II) of normal rat serum obtained by gel filtration when separated by vertical discontinuous polyacrylamide-gel electrophoresis.

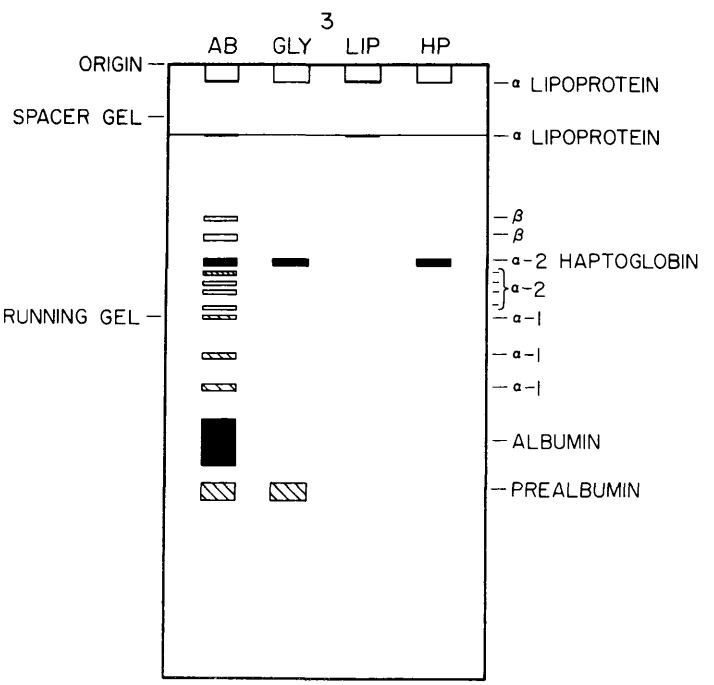

FIGURE 4. Diagrammatic representation of the low-molecular-weight fraction (III) of normal rat serum obtained by gel filtration when separated by vertical discontinuous polyacrylamide-gel electrophoresis.

in the alpha-2 range. Lipoprotein stain (LIP) is present at the origin and at the gel interface. A benzidine-positive band (HP) is visible in the alpha-2 region corresponding to the glycoprotein band found here.

The high-molecular-weight fractions of both human and rat serum showed similarities in the gamma region (HI and RI in fig. 5). Human serum showed two alpha-2 globulins, while rat serum showed these globulins in the alpha-1 range. The intermediate-molecular-weight fraction of human serum had a more diffuse gamma region that did that of the comparable rat fraction (HII, RII). Both human and rat serum had alpha globulins in the intermediate fraction. The low-molecular-weight fractions of both human and rat serum (HIII, RIII) had comparable bands in the albumin, alpha, and beta regions. A fourth human fraction (HIV) showed a slow alpha-2 band, but was mainly albumin.

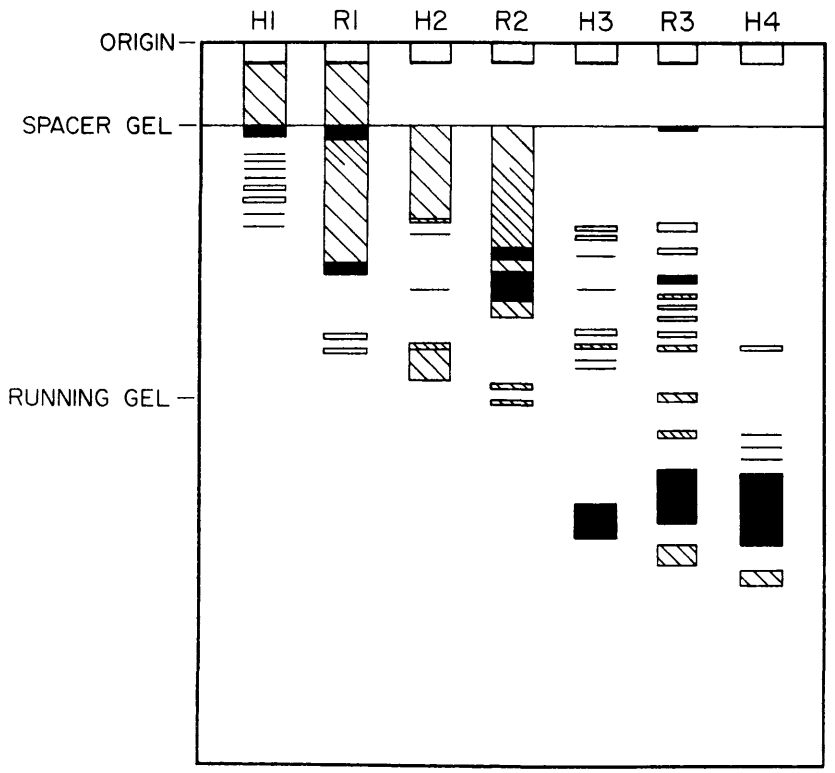

FIGURE 5. Diagrammatic comparison of the three fractions of normal rat serum and the four fractions of human serum obtained by gel filtration when separated by vertical discontinuous polyacrylamide-gel electrophoresis.

DISCUSSION

Of the three fractions of rat serum, the low-molecular-weight fraction is most critical, since the granulocyte maturation factor was found here (Graham and Morrison, 1970). Robinson (1969) has isolated an alpha glycoprotein (about 60,000 MW) from human blood and urine which stimulated production of mature granulocytes in normal and leukemic blast-cell tissue culture.

The low-molecular-weight fraction of normal rat serum had a range in molecular weight of approximately 40,000 to 150,000, based on Sephadex sieving. General staining showed 14 reproducible bands (fig. 4). Mobility classification of rat-

serum proteins was based on comparison with human-serum proteins fractionated in an identical manner. A band that migrated ahead of the other components and gave a positive glycoportein stain corresponded to that of the prealbumin of human serum (Sandor, 1966). This was followed by the major component of rat serum, albumin, which makes up 59.1% of the total protein content of this serum (Engle and Woods, 1960).

The globulin components showed three points of specific staining. One slow globulin gave a positive glycoprotein stain and a benzidine-positive band indicative of haptoglobin, and of alpha-2 glycoprotein similar to that of human serum (Sandor, 1966). Positive lipoprotein staining was found at the origin and at the spacer gel-running gel interface.

Five alpha-2 globulins and three alpha-1 globulins were demonstrated to be present in the low-molecular-weight fraction of rat serum. Based on Robinson's (1969) molecular-weight determination, only the three alpha-1 globulins with fastest mobility could function as the "maturation factor". *In vivo* tests of this hypothesis are in progress. Two bands followed the benzidine-positive haptoglobin band in rat serum. These corresponded to human beta globulins having an electrophoretic mobility slower than haptoglobin and a higher molecular weight.

The components of the intermediate-molecular-weight fraction included proteins with a molecular weight range of approximately 150,000 to 300,000 as determined by molecular sieving. The globulins in this group moved in the alpha and gamma ranges (fig. 3). Those found in the running gel were glycoproteins. Another benzidine-positive glycoprotein was found in this fraction and may represent a polymer of haptoglobin. Its presence in other sera in a polymerized form has been noted (Putnam, 1965; Winzler, 1960). Based on the gel-filtration separation, this was probably the dimer having a molecular weight of about 170,000.

The gamma globulins of this fraction were represented by two definite bands, although the region showed a diffuse staining with both general and glycoprotein stains. These represented the 7S gamma globulins and are comparable to those found in human serum of molecular weight 157,000 (Muller-Eberhard, Kunkel, and Franklin, 1956; Putnam, 1965). The origin band gave a positive stain for lipoprotein.

The molecular weight of proteins in the high-molecular-weight fraction was above 300,000, based on molecular sieving. Proteins in this group were found in the alpha and gamma regions and at the origin (fig. 2). Those in the running gel are comparable to the alpha-2 and gamma macroglobulins of human serum (Clarke, 1964; Davis, 1964; Kunkel, 1960). Graham (1969) reported the presence of two alpha-1 and two gamma macroglobulins in rat serum. An alpha-1 macroglobulin has also been reported in rabbit serum (Svehag, Chesebro, and Bloth, 1967). This indicates the species-variable mobility of the alpha macroglobulin. The current work showed a third diffuse band in the gamma region of rat serum. Both alpha and gamma groups showed a positive glycoprotein stain indicative of their ability to bind carbohydrate (Winzler, 1960).

SUMMARY

The study of the leukemias and other blood diseases has been complicated by the complex kinetics of hemopoiesis. The inability to isolate pure preparations of suspected "control factors" in granulo poiesis has been one major difficulty. The present study was concerned with the relative mobilities and distribution of the identifiable protein components of rat serum. Mobility classification of rat serum proteins was based on comparison with human serum proteins fractionated in an identical manner by vertical discontinuous polyacrylamide gel electrophoresis.

The low-molecular-weight fraction had a prealbumin, an albumin, three alpha-1 globulins, five alpha-2 globulins, two beta globulins, and two lipoprotein bands of the high-density class. The slowest alpha-2 globulin gave positive glycoprotein

and haptoglobin stains, while the prealbumin gave a positive glycoprotein reaction. The three alpha-1 globulins in this fraction are implicated as a "factor" controlling the maturation of granulocytes in rat bone-marrow.

The intermediate- and high-molecular-weight fractions contained mainly alpha and gamma glycoproteins. Both fractions also contained lipoproteins, the alpha high-density band being located in the intermediate-molecular-weight fraction. The benzidine-positive band in the intermediate-molecular-weight fraction was indicative of the polymer form of haptoglobin.

LITERATURE CITED

Clark, J. M. 1964. Experimental biochemistry. W. H. Freeman, San Francisco. 228 p.

Clarke, J. T. 1964. Simplified "disc" (polyacrylamide gel) electrophoresis. Ann. N. Y. Acad. Sci. 121: 428–436.

Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. Ann. N. Y. Acad. Sci. 121: 404–427.

Dolezalova, V., Z. Brada, and **A. Kocent.** 1965. The behavior of some proteins of normal rat serum studied by gel filtration and electrophoresis. Biochem. Biophsy. Acta. 107: 294–306.

E-C Apparatus. 1966. Discontinuous vertical gel electrophoresis using the E–C vertical gel electrophoresis system. E–C Bulletin 2(1). 32 p.

Engle, R. L., Jr., and **K. R. Woods.** 1960. Comparative biochemistry and embryology, 183–265. *In* F. W. Putnam, editor, The plasma proteins. Vol. 2. Academic Press, New York. 518 p.

Ferris, T. G., R. E. Easterling, and **R. E. Budd.** 1963. Determination of haptoglobin types by electrophoresis in acrylamide gel. Clin. Chim. Acta. 8: 792–793.

Graham, J. D. 1969. Immunologic assay for granulopoietic factors in rat serum. Unpublished Ph.D. dissert. Kent State Univ. 152 p. Abstr. Univ. Microfilms 70–5969. Ann Arbor, Michigan.

Graham, J. D., J. H. Morrison, and **J. R. Toepfer.** 1969. Use of Myleran in the study of leukopoiesis and leukopoietic factors. Intern. Symp. on Genetic Effects of Radiation and Radiomimetic Chemicals. Japan. J. Genet. 44(Supl. 2): 91–93.

Graham, J. D., and **C. J. McMahon.** 1971. Granulocyte maturation factor: control of granulopoiesis in bone marrow of normal albino rats. Trans. Am. Microscop. Soc. 90: 238–242.

Graham, J. D., and **J. H. Morrison.** 1970. Immunologic assay for factors controlling granulopoiesis in rat bone marrow. I. Isolation of a maturation factor from rat serum. Trans. Amer. Microscop. Soc. 89: 434–442.

Keyser, J. W. 1964. Staining of serum glycoproteins after electrophoretic separation in acrylamide gels. Anal. Biochem. 9: 249–252.

Kunkel, H. G. 1960. Macroglobulins and high molecular weight antibodies, 279–307. *In* F. W. Putnam, editor, The plasma proteins. Vol. 1. Academic Press, New York. 420 p.

Muller-Eberhard, H. J., H. G. Kunkel, and **E. C. Franklin.** 1956. Two types of gamma globulin differing in carbohydrate content. Proc. Soc. Exp. Biol. Med. 93: 146–150.

Peacock, A. C., S. L. Bunting, and **K. G. Queen.** 1965. Serum protein electrophoresis in acrylamide gel: Patterns from normal human subjects. Science 147: 1451–1453.

Phelps, R. A., and **F. W. Putnam.** 1960. Chemical composition and molecular parameters of purified plasma proteins, 143–178. *In* F. W. Putnam, editor, The plasma proteins. Vol. 1. Academic Press, New York. 420 p.

Putnam, F. W. 1965. Structure and function of the plasma proteins, 153–267. *In* H. Neurath, editor, The proteins. Vol. 3. Academic Press, New York. 585 p.

Raymond, S., J. L. Miles, and **J. C. J. Lee.** 1966. Lipoprotein patterns in acrylamide gel electrophoresis. Science 151: 346–347.

Robinson, W. A., D. Metcalf, and **T. R. Bradley.** 1967. Stimulation by normal and leukemic mouse sera of colony formation *in vitro* by mouse bone marrow cells. J. Cell. Physiol. 69: 83–90.

Sandor, G. 1966. Serum proteins in health and disease. Chapman and Hall, London. 768 p.

Svehag, S. E., B. Chesebro, and **B. Bloth.** 1967. Ultrastructure of gamma M immunoglobulin and alpha macroglobulin: Electronmicroscopic study. Science 158: 933–936.

Winzler, R. J. 1960. Glycoproteins, 309–347. *In* F. W. Putnam, editor, The plasma proteins. Vol. 1. Academic Press, New York. 420 p.